Germline *MLH1* and *MSH2* mutational spectrum including frequent large genomic aberrations in Hungarian hereditary non-polyposis colorectal cancer families: Implications for genetic testing

Janos Papp, Marietta E Kovacs, Edith Olah

Janos Papp, Marietta E Kovacs, Edith Olah, Department of Molecular Genetics, National Institute of Oncology, Rath Gy. u. 7-9, H-1122 Budapest, Hungary

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Co-first-author: Janos Papp and Marietta E Kovacs

Correspondence to: Dr. Edith Olah, Department Molecular Genetics, National Institute of Oncology, Rath Gy. u. 7-9, H-1122 Budapest, Hungary. e.olah@oncol.hu

Telephone: +36-1-2248788 Fax: +36-1-2248708

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Abstract

AIM: To analyze the prevalence of germline *MLH1* and *MSH2* gene mutations and evaluate the clinical characteristics of Hungarian hereditary non-polyposis colorectal cancer (HNPCC) families.

METHODS: Thirty-six kindreds were tested for mutations using conformation sensitive gel electrophoreses, direct sequencing and also screening for genomic rearrangements applying multiplex ligation-dependent probe amplification (MLPA).

RESULTS: Eighteen germline mutations (50%) were identified, 9 in *MLH1* and 9 in *MSH2*. Sixteen of these sequence alterations were considered pathogenic, the remaining two were non-conservative missense alterations occurring at highly conserved functional motifs. The majority of the definite pathogenic mutations (81%, 13/16) were found in families fulfilling the stringent Amsterdam I/II criteria, including three rearrangements revealed by MLPA (two in *MSH2* and one in *MLH1*). However, in three out of sixteen HNPCC-suspected families (19%), a disease-causing alteration could be revealed. Furthermore, nine mutations described here are novel, and none of the sequence changes were found in more than one family.

CONCLUSION: Our study describes for the first time the prevalence and spectrum of germline mismatch repair gene mutations in Hungarian HNPCC and suspected-HNPCC families. The results presented here suggest that clinical selection criteria should be relaxed and detection of genomic rearrangements should be included in genetic screening in this population.

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) is the most common form of hereditary colorectal cancer (CRC), accounting for 1%-5% of cases. This syndrome is characterized by early onset CRC and endometrial cancer, although the incidence of other malignant tumours, e.g. cancers of the stomach, urothelium, small bowel and ovary, is also increased in HNPCC patients^[1].

HNPCC is autosomal dominantly inherited and is associated with germline mutations in at least five mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, *PMS1* and *PMS2*)^[2]. The identification of carriers is usually based on screening individuals from families fulfilling international criteria for the syndrome, namely Amsterdam criteria I or II^[3,4] or less stringent criteria referred to as the Bethesda guidelines^[5].

Mutations in *MLH1* (MIM# 120436) and *MSH2* (MIM# 120435) are considered the major cause of HNPCC, since germline alterations of these genes have been found to be responsible for more than 90% of mutation carrier HNPCC families. According to the database of the International Society for Gastrointestinal Hereditary Tumors (InSiGHT; <http://www.insight-group.org>), currently more than 450 different pathogenic mutations have been described in these genes, accounting for approximately 750 HNPCC kindreds worldwide^[6].

The majority of reported *MLH1* and *MSH2* mutations are nonsense, missense, or frameshift mutations as well as changes affecting splice sites. However, more recently, utilization of new techniques has led to the realization

that in some populations a relatively large proportion of pathogenic alterations are genomic rearrangements, in most cases single or multi-exonic deletions or duplications inactivating *MLH1* and/or *MSH2*^[7,8]. The known mutations are scattered throughout the coding regions of these genes, although, in particular populations (e.g. in Finland, Poland and in the United States), some recurrent changes have been found and described as founder mutations^[9-11].

Predictive genetic testing for germline mutations in MMR genes can allow identification of HNPCC families, and are thus of great importance for counselling, surveillance and management of at-risk patients. Nevertheless, since the reliability of the family history provided by patients is often incomplete and questionable, and because germline mutations are occasionally identified even in atypical HNPCC families^[12], the requirements for establishing objective strategies of genetic testing are pressing. Therefore, assessing the prevalence and spectrum of *MLH1* and *MSH2* mutations in afflicted families in a given population is an essential first step in improving diagnosis of HNPCC, thus identifying individuals at high risk of colorectal and endometrial cancer.

MATERIALS AND METHODS

Patients and controls

From 1994 to 2004, clinical data and samples from 36 Hungarian HNPCC families were collected, of which 20 fulfilled the Amsterdam I - II criteria and 16 were classified as 'pedigrees suggestive of HNPCC'. Although they did not meet internationally defined inclusion criteria, a suspicion of HNPCC was raised on the basis of their personal or family history being close to at least one of the modified Amsterdam criteria or the Bethesda guidelines^[13]. The study was approved by the Institutional Ethical Board, and written informed consent was obtained from each patient following genetic counselling. In addition, 63 healthy subjects from the same population were analyzed for the presence of mutations with uncertain clinical significance.

Mutation analysis

Blood samples were obtained from all consenting subjects and DNA was extracted with the classic phenol-chloroform method. The entire *MLH1* and *MSH2* coding region and splice junctions were amplified by PCR from genomic DNA using primer sequences published earlier^[14,15]. Systematic screening for point mutations in both genes was performed using conformation-sensitive gel electrophoreses (heteroduplex analysis and single-strand conformation polymorphism analysis) on all amplicons^[16,17]. All samples showing altered migration patterns were subjected to direct bi-directional sequencing using an ABI 310 genetic analyzer (Applied Biosystems).

Genomic aberration screening

In addition to standard mutation analysis, both *MLH1* and *MSH2* coding regions were screened for genomic rearrangements using the multiplex ligation-dependent probe amplification (MLPA) technique^[18]. In brief, 150-200

ng of genomic DNA in 5 μ L Tris-EDTA was denatured, cooled, and incubated with the probe mix containing one probe for each *MLH1/MSH2* exon and several control probes specific for sequences outside these genes. After probe hybridization, ligation using ligase-65 enzyme was done at 54°C for 15 min. Ligation products were then amplified by PCR using one fluorescently labelled primer. The PCR products were separated and analyzed with an ABI 310 genetic analyzer (Applied Biosystems). Peak heights for each fragment were compared to those of a control sample and deletions/duplications were suspected when peak intensities differed by more than 30%.

Since small sequence changes (SNPs, small insertions or deletions) close to the ligation site may also alter the peak size in MLPA, all exons showing copy number changes were directly sequenced. The presence of genomic rearrangements was confirmed by long PCR using the Expand Long Template PCR System (Roche) or Hercules Hotstart DNA polymerase (Stratagene) according to the manufacturers' instructions. The PCR products were separated and sized by agarose gel electrophoresis and visualized by ethidium-bromide staining.

The mutation nomenclature used here complies with the recommendations of den Dunnen and Antonarakis^[19,20]. Sequence variations were named in relation to the ATG codon in cDNA reference sequences (NM000249.2 and NM000251.1 for *MLH1* and *MSH2*, respectively), while changes at the amino acid level were deduced from nucleotide alterations in relation to the protein reference sequences NP00240.1 for *MLH1* and NP00242.1 for *MSH2*. All variants were ascertained by a replicate experiment.

RESULTS AND DISCUSSION

Mutation analysis of affected members from 20 Hungarian HNPCC and 16 suspected HNPCC families revealed 18 germline alterations in 18 unrelated probands (50%), 9 in *MLH1* and 9 in *MSH2*. Based on a literature review and checking the variants listed in the InSiGHT mutation database, we found that 9 out of 18 mutations have not been described previously. Sixteen of the sequence alterations were considered as pathogenic mutations either on the basis of published data or due to their predicted deleterious effects on the *MSH2* or *MLH1* protein such as insertions/deletions, nonsense changes, splice-site mutations and genomic rearrangements. The remaining two variants were missense alterations occurring at highly conserved motifs, one in *MLH1* and the other in *MSH2*. Thirteen of the definite pathogenic mutations were found in members of HNPCC families fulfilling the stringent Amsterdam I / II criteria (including two deletions in *MSH2* and one in *MLH1*). However, in three out of sixteen (19%) HNPCC-suspected families, a disease-causing alteration could be revealed, perhaps demonstrating the need for different inclusion decisions for this population. The MMR gene mutations, as well as the medical and family histories of patients, are shown in Table 1.

Previously identified pathogenic mutations

Nine of the pathogenic changes identified in Hungarian

Table 1 Characteristics of carrier families and Hungarian MMR gene mutations

Proband ID	Proband phenotype ¹	Family phenotype ²	Gene	Exon	Nucleotide position of the mutation	Effect/Consequence of the mutation
HFC003	C25	A I:	<i>MLH1</i>	2	c.187G>A	p.D63N
HFC077	C53	A I:	<i>MLH1</i>	16	c.1852_1854del	p.K618del
HFC100	C39	A I:	<i>MLH1</i>	18-19	c.2078_2172del	p.E693_K724delAfsX8
HFC075	C33	A I:	<i>MSH2</i>	1-2	c.1-?_c.366+?del	no protein?
HFC048	C31	A I:	<i>MSH2</i>	3-7	c.367-?_c.1226+?del	deletion of exons 3-7
HFC066	C22	A I:	<i>MSH2</i>	4	c.759_762del	p.M253_N254delIfsX20
HFC108	C30	A I:	<i>MSH2</i>	7	c.1226_1227del	p.Q409RfsX7
HFC014	C29	A I:	<i>MSH2</i>	10	c.1661+1G>T	splice defect?
HFC021	C47	A I:	<i>MSH2</i>	13	c.2131C>T	p.R711X
HFC050	C36	A II:	<i>MLH1</i>	8	c.677G>A	p.R226Q
HFC045	C19	A II:	<i>MLH1</i>	13	c.1489_1490insC	p.R497fsX6
HFE063	E47	A II:	<i>MLH1</i>	16	c.1875T>G	p.Y625X
MPX050	C36	A II:	<i>MLH1</i>	19	c.2141G>A	p.W714X
HFC017	C27	A II:	<i>MSH2</i>	12	c.1861C>T	p.R621X
HFC072	C35	SH:	<i>MLH1</i>	13	c.1411_1414del	p.K471_R472delDfsX19
HFC104	C39	SH:	<i>MLH1</i>	16	c.1852_1853delinsGC	p.K618A
HFC078	C53	SH:	<i>MSH2</i>	1	c.146A>T	p.D49V
HFC074	C45, 50, 55	SH:	<i>MSH2</i>	15	c.2620_2621ins115	p.Y874CfsX3

¹Cancer type and age at onset (C: colorectal; E: endometrial); ²Categories according to the Amsterdam criteria (A I: Amsterdam I; A II: Amsterdam II; SH: suspected HNPCC) and number and type of cancer in the family (C: colorectal; E: endometrial; S: stomach; I: intestinal). Novel mutations are in bold.

families were found in the InSiGHT mutation database. Six of these were either nonsense mutations or small deletions, resulting in a premature stop codon predicted to be a subject of rapid mRNA degradation through nonsense-mediated decay (NMD)^[21] or leading to the production of a truncated protein. One alteration (c.677G>A, p.R226Q), affecting the last nucleotide of *MLH1* exon 8, presumably leads to splicing aberration^[22]. This latter mutation was revealed in the proband of our family HFC050, diagnosed with colorectal carcinoma at age 36. Cosegregation with the disease phenotype could not be assessed.

The remaining two mutations were found in exon 16 of *MLH1* (c.1852_1854del, p.K618del and c.1852_1853delinsGC, p.K618A), both affecting the same nonconserved lysine residue. Although K618A is one of the few changes listed as pathogenic and also as non-pathogenic in the InSiGHT database, previously published data indicate that both of these alterations are associated with an increased risk of colorectal cancer^[23-25]. Furthermore, this C-terminal part of the MLH1 protein was shown to play a crucial role in protein-protein interactions, and a mutation of the same amino acid, K618T, caused nearly complete disruption of the interaction between MLH1 and MRE11, perhaps highlighting the potential function of this residue^[26]. In our study, the non-conservative K618A variant was found in a small family (HFC104) showing two cases with early onset CRC (diagnosed at ages of 39 and 47 years) who were second degree relatives. DNA was available from only one of them, so cosegregation could not be evaluated. The in-frame deletion K618del was revealed in a family meeting the stringent Amsterdam I criteria (HFC077), but DNA sample was available only from the proband (diagnosed with CRC at age of 53).

None of these variants was present in 126 control chromosomes and also, no other possibly damaging sequence variant was found in any of these cases, further

supporting the pathogenicity of these alterations.

Novel pathogenic mutations

Out of the nine novel sequence changes identified in Hungarian families, seven were considered pathogenic.

The 1661+1G>T substitution, found in a family fulfilling the Amsterdam I criteria (HFC014), could dramatically decrease the information content of the canonical 5' splice site in intron 10 of *MSH2* ($\Delta R_i = -12.8$), as determined by automated splicing mutation analysis (<http://splice.cmh.edu>)^[27]. Although an RNA sample from the carrier proband was not available to test this hypothesis, the mutation is predicted to cause skipping of exon 10, thus leading to a frameshift and a premature stop codon.

In family HFE063, the proband was diagnosed with endometrial cancer at the age of 47. Mutation analysis revealed a substitution in exon 16 of *MLH1*. Sequencing showed this to be a c.1875T>G transversion, resulting in an immediate stop codon (p.Y625X). The mutation was also identified in the proband's brother.

The proband of family HFC066 was diagnosed with cancer of the colorectum at the unusually early age of 22. His father died of CRC at the age of 32, and 5 other family members were affected with colorectal cancer (ages from 30 to 52 years). Conformation-sensitive mutation screening revealed a small deletion in exon 4 of *MSH2*. Sequence analysis identified the c.759_762delGAAT mutation, which results in frameshift and creates a stop 20 codons downstream. The mutation was also identified in the proband's grandfather (CRC at the age of 52).

Family HFC074 was selected for mutation screening because, although the family phenotype did not fulfil Amsterdam criteria I / II, the proband was diagnosed with three metachronous colorectal cancers (at the age of 45, 50 and 55 years) and his father was also affected (CRC at the age of 62). Testing for large genomic changes using

Figure 1 Deletion of *MSH2* exons 3-7 in patient HFC048. **A:** MLPA chromatogram is shown for a negative control (above) and patient HFC048 (below). The peaks for *MSH2* exons are grey, the *MLH1* exons and the control peaks are blank. The numbering of *MSH2* exons is given between the panels, the exons deleted in patient HFC048 are marked by arrows; **B:** Long PCR amplification of the deleted region. The sense primer (5'-gctggaaataaggcatcaag-3') is located in exon 2, the antisense primer (5'-aggagtcacaaaaactgccaac-3') in exon 8, approximately 36 kb apart on the *MSH2* genomic sequence. Lane "M" contains a molecular weight marker (BenchTop 1kb DNA Ladder, Promega), arrow marking the 6 kb band. Lane 1 shows that amplification from a negative control sample is not successful, while a 6 kb product can be amplified from the positive sample (Lane 2), indicating the presence of a 30 kb deletion.

MLPA analysis revealed a partial duplication of exon 15 of *MSH2* following insertion of a guanine nucleotide. This aberration causes a frameshift, thus predicted to result in rapid degradation of RNA due to the nonsense-mediated decay surveillance mechanism.

MLPA screening of the proband (CRC diagnosed at the age of 33 years) from the Amsterdam I family HFC075 revealed a large genomic deletion affecting exons 1 and 2 of *MSH2*. The 5' breakpoints of deletions are known to be very diverse^[28,29]. In our case, the breakpoints for this deletion were not determined. However, since the deletion removes the transcription start site of the gene, it is predicted to result in a null allele.

In the proband of our large Amsterdam I family HFC048, a large genomic deletion of *MSH2* was detected using the MLPA method. Although the exact breakpoints have not been identified yet, long PCR analysis showed an approximately 30 kb deletion (Figure 1). This mutation is predicted to cause an out-of-frame deletion of exons 3-7, the resulting premature stop codon is presumably subjected to NMD.

Finally, MLPA analysis of family HFC100, a large kindred fulfilling Amsterdam I criteria, showed a genomic deletion of *MLH1*, starting within exon 18 (codon 693) and ending in exon 19 (codon 724), thus removing parts of these exons and also the intervening intronic sequences. The result of this aberration is a deletion of 31 residues,

Table 2 Frequency of pathogenic or unclassified sequence variants in Hungarian HNPCC and suspected HNPCC families

	Frequency of variant (%)		
	Pathogenic	Unclassified	Total
HNPCC	13/20 (65)	1/20 (5)	14/20 (70)
(Amsterdam I + II)			
Suspected HNPCC	3/16 (19)	1/16 (6)	4/16 (25)
Total	16/36 (44)	2/36 (6)	18/36 (50)

and the appearance of a new frame terminating 8 residues downstream. Since this aberration removes the last exon-exon junction, nonsense-mediated decay cannot affect the stability of RNA, a slightly shorter protein is predicted to appear. The pathogenicity of this mutation is supported by the fact that (a) this most C-terminal part of *MLH1* was shown to play a crucial role in protein-protein binding^[26], and (b) in our family the mutation was also found in the proband's mother affected by multiple primary cancers of the colon and stomach.

Novel variants with assumed pathogenicity

In family HFC003, the identified mutation is a single base substitution in exon 2 of the *MLH1* gene. This mutation, c.187G>A, converts the aspartic acid residue at codon 63 to an asparagine (p.D63N) at the ATP binding pocket of the *MLH1* protein. The substituted residue is highly conserved in a diverse class of ATPases, containing also type II topoisomerase, histidine kinases, gyrase B and Hsp90. In an earlier study, this p.D63N mutation was generated using site-directed mutagenesis and was shown to result not only in decreased activity but also in a dramatically reduced expression of the MutL-alpha complex containing the variant protein due to rapid degradation^[30]. In our family, c.187G>A found in two CRC cases (father and son, diagnosed with CRC at the ages 40 and 25 years, respectively) was cosegregating with the disease, but was missing from seven other family members, who were asymptomatic at the age ranging from 19 to 57 years. These data, together with the findings mentioned above, strongly support the pathogenic nature of this mutation.

Additionally, in HFC078, a small family not fulfilling any of the accepted criteria (only one clinically confirmed colorectal cancer diagnosed at the age of 53 years), another substitution was revealed by mutation pre-screening and sequence analysis. The c.146A>T (p.D49V) transversion is situated in an N-terminal motif which is highly conserved in all MutS proteins throughout evolution. Mutations in this motif (GXFY(X)S₂DA) have been shown to play a crucial role in mismatch binding activity^[31,32]. Unfortunately, since cosegregation with the disease could not be assessed in our atypical family, more data are needed to confirm or refute the pathogenicity of this variant.

Although none of these variants was found in 126 control chromosomes, these mutations are not to be used for predictive diagnostics, but considered unclassified variants until further functional tests prove or refute their causal association with the disease phenotype.

The current study, representing the first molecular

characterization of HNPCC in Hungarian families, has expanded the known mutation spectrum of *MSH2* and *MLH1* genes. In summary, a total of 18 germline mutations were identified in 36 Hungarian cases (14 in HNPCC and 4 in suspected HNPCC families), half of them being novel (Table 2).

On the basis of the diffuse spectrum of point mutations and the relatively high frequency of genomic changes, we conclude that screening for rearrangements and mutations in the entire coding regions of these genes should be considered in this population. Moreover, since 25% of the suspected Hungarian HNPCC families were shown to carry a mutation in one of the MMR genes (three definitive pathogenic mutations and one mutation for which pathogenicity is assumed), we suggest that not only families fulfilling the stringent Amsterdam criteria, but also those following more relaxed guidelines should undergo genetic testing.

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